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Amplification of the Housekeeping Sigma Factor in *Pseudomonas fluorescens* CHA0 Enhances Antibiotic Production and Improves Biocontrol Abilities

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***Pseudomonas fluorescens* CHA0 produces a variety of secondary metabolites, in particular the antibiotics pyoluteorin and 2,4-diacetylphloroglucinol, and protects various plants from diseases caused by soilborne pathogenic fungi. The *rpoD* gene encoding the housekeeping sigma factor σ^{70} of *P. fluorescens* was sequenced. The deduced RpoD protein showed 83% identity with RpoD of *Pseudomonas aeruginosa* and 67% identity with RpoD of *Escherichia coli*. Attempts to inactivate the single chromosomal *rpoD* gene of strain CHA0 were unsuccessful, indicating an essential role of this gene. When *rpoD* was carried by an IncP vector in strain CHA0, the production of both antibiotics was increased severalfold and, in parallel, protection of cucumber against disease caused by *Pythium ultimum* was improved, in comparison with strain CHA0.**

Antibiotic compounds produced by root-colonizing fluorescent pseudomonads play a significant role in the suppression of soilborne diseases of important crop plants (6, 7, 30). During stationary growth phase, various *Pseudomonas* biocontrol strains synthesize a range of antibiotics, such as phenazine carboxylic acid (PCA) (43), pyrrolnitrin (16, 18), oomycin A (13), 2,4-diacetylphloroglucinol (Phl) (10, 21, 45), and pyoluteorin (Plt) (19, 32). In several studies, it has been demonstrated that antibiotic-negative mutants of *Pseudomonas* strains have a reduced ability to suppress root diseases, compared with the biocontrol effects of the parental strains. For instance, a mutant of *Pseudomonas fluorescens* CHA0 affected in the synthesis of Phl gives a diminished protection of tobacco against black root rot caused by the fungus *Thielaviopsis basicola* (21, 23). A pyrrolnitrin-defective mutant of *P. fluorescens* BL915 no longer suppresses *Rhizoctonia solani*-induced damping-off of cotton (16), and phenazine-negative derivatives of fluorescent pseudomonads lack part of their ability to protect wheat from take-all disease (35, 43). When antibiotic production in these mutants is restored by complementation or recombination, the plant-beneficial effects also return to normal. The antibiotic compounds may antagonize the plant-pathogenic fungi either directly, by inhibiting them on the roots, or indirectly, by stimulation of plant defence mechanisms (46, 49, 50).

The biocontrol performance of soil pseudomonads may be improved by the introduction of antibiotic biosynthetic genes (7). A recombinant cosmid expressing the *phl* structural genes of *Pseudomonas aureofaciens* Q2-87 was transferred to *P. fluorescens* 2-79, which naturally produces PCA (45). The recombinant strain had increased antifungal activity in vitro against *Gaeumannomyces graminis* var. *tritici*, *Pythium ultimum*, and *R. solani*. The PCA biosynthetic genes of *P. fluorescens* 2-79 were introduced into different PCA-nonproducing fluorescent pseudomonads, and the recombinant strains proved more inhibitory to *G. graminis* var. *tritici* in vitro and in vivo than the wild type (14).

The global regulator GacA is essential for antibiotic production in *P. fluorescens* (27). GacA is a response regulator in the FixJ family of two-component systems. A cognate sensor has tentatively been identified (30); it is closely related to LemA, a sensor protein controlling toxin and protease expression in *P. syringae* (20). Additional controls operate on individual antibiotic pathways. Phenazine biosynthetic genes in *P. aureofaciens* are regulated by the LuxR-like transcriptional activator PhzR, in response to high cell densities (35).

In previous work, we have demonstrated that pME3090, a cosmid carrying a 22-kb chromosomal fragment of *P. fluorescens* CHA0 (40), enhances severalfold the production of Phl and Plt in the wild type (32). The antibiotic-overproducing strain shows improved biocontrol abilities in several host-pathogen systems (32). However, it was not clear whether the effects observed were due to one gene or possibly to several different genes carried by pME3090. We report here that both enhanced antibiotic production and improved protection against damping-off of cucumber are due to amplification of a single gene encoding the housekeeping sigma factor σ^{70}.

Determination of a minimal-size fragment required for antibiotic overproduction in *P. fluorescens* CHA0. To localize the gene(s) mediating Phl and Plt overproduction in pME3090, we performed subcloning experiments, which led to a 7-kb *Bgl*II-*Hin*dIII fragment in the pVK100 derivative pME3402 (Table 1). Then, insertions of transposon Tn*1737*Cm were obtained in the insert of plasmid pME3402. Transposon mutagenesis was carried out with *Escherichia coli* JM108 (51) carrying a chromosomally integrated Tn*1737*Cm, which had been introduced from plasmid pRU886 (44). Strain JM108::Tn*1737*Cm containing pME3402 was spread on chloramphenicol gradient (0→1,000-μg/ml) plates. Colonies resistant to ≥500 μg/ml carried Tn*1737*Cm in pME3402. One construct, pME3402::Tn*1737*Cm-8, was not able to enhance Phl and Plt production in strain CHA0 (Table 1), whereas 39 other Tn*1737*Cm insertions did not affect antibiotic overproduction. Tn*1737*Cm carries an *Eco*RI site in each terminal inverted repeat (44). The

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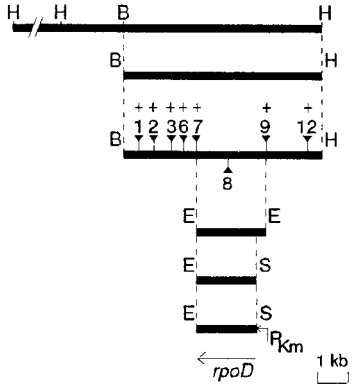
TABLE 1. Extracellular concentrations of Plt, Phl, and Mphl produced by *P. fluorescens* CHA0 and its derivatives

Bacterial strain	Plasmid	DNA fragments derived from pME3090[a]	Overproduction of Plt[b]	King's medium B agar[c]	Malt agar[c]	
				Plt[d]	Phl[d]	Mphl[d]
CHA0	None		-	3.9 ± 2.6	1.5 ± 0.5	< 0.1
	pME3090		+	27.0 ± 1.9	8.8 ± 3.4	1.8 ± 0.7
	pME3402		+	nd	nd	nd
	pME3402::Tn*1737*Cm		+	nd	nd	nd
			-	3.3 ± 3.8	2.0 ± 0.3	0.2 ± 0.0
	pME3415		+	nd	nd	nd
	pME3424		+	26.6 ± 7.0	4.9 ± 0.4	1.1 ± 0.1
	pME3423		+	25.7 ± 11.6	5.8 ± 1.4	1.1 ± 0.3

[a] Derivatives of pVK100 were mobilized from *E. coli* to *P. fluorescens* with the helper pME497 (47). Restriction sites: B, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; S, *Sac*I. The numbered symbols ↑ indicate the locations of Tn*1737*Cm insertions in pME3402. ←, orientation of the kanamycin promoter (P_{Km}).

[b] The overproduction of Plt by strain CHA0 carrying pME3090 or its derivatives was assessed qualitatively by measuring growth inhibition of *E. coli* K-12. Derivatives of *P. fluorescens* CHA0 obtained during subcloning experiments were spotted on King's medium B plates at 1 cm from the edge. After incubation of the plates at 23°C for 3 days, the cells were irradiated with UV light with a germicidal lamp for 5 min. Each plate was overlaid with 4 ml of an overnight culture of *E. coli* K-12 diluted 1/10 in soft agar (nutrient yeast broth containing 0.8% agar) and incubated at 37°C for 1 day. Derivatives of *P. fluorescens* overproducing Plt and Phl gave clear zones of growth inhibition of *E. coli* K-12, whereas the wild-type CHA0 did not show inhibition. + and −, inhibition and no inhibition of *E. coli* K-12 growth, respectively.

[c] The bacteria were grown in King's medium B agar at 24°C and on malt agar plates at 18°C for 3 days. The average numbers of bacteria per plate at the time of extraction did not vary significantly with different strains and reached ≈3×10^{10} cells per malt agar plate and ≈10^{11} cells per King's medium B plate. The agar plates were extracted as described elsewhere (21, 32). Each value is the mean ± standard error of the mean of three independent experiments with two plates per experiment.

[d] Antibiotic production (in microliters per milliliter of medium).

*Eco*RI sites of plasmids pME3402::Tn*1737*Cm-7 and pME3402::Tn*1737*Cm-9 (Table 1) were used to generate a 2.3-kb *Eco*RI fragment forming the insert of pME3415. Further subcloning produced plasmid pME3424 carrying a 2.0-kb *Sac*I-*Eco*RI insert (Table 1). Since it was not certain whether this 2.0-kb fragment would contain the natural promoter(s), it was also inserted downstream of the constitutive kanamycin resistance promoter of the vector pVK100, resulting in pME3423 (Table 1). Strain CHA0 carrying either pME3424 or pME3423 overproduced Plt about sevenfold on King's medium B and Phl about threefold on malt agar, compared with strain CHA0 (Table 1). Thus, the vector promoter of pME3423 had no effect, and it is likely that the cloned 2.0-kb fragment contains at least one promoter. Monoacetylphloroglucinol (Mphl), a precursor of Phl (7), was accumulated in small quantities on malt agar. The relative amounts of Mphl paralleled those of Phl in various strains (Table 1).

Nucleotide sequence and expression of the *rpoD* gene encoding σ^{70} of *P. fluorescens* CHA0. The nucleotide sequence of the 2,326-bp *Eco*RI fragment in pME3415 on both strands by the chain termination technique (17) was determined by using [α-^{32}P]dATP, 7-deaza-dGTP, and Sequenase version 2.0 (U.S. Biochemical Corp.). One large open reading frame (ORF) of 615 codons is apparent (Fig. 1), and its deduced product has a calculated molecular mass of 69,436 Da. Upstream of the presumed ATG start codon, there is a sequence (GGATAG) which could act as a ribosome-binding site. Downstream of the TAA termination codon, inverted repeats followed by a run of five T residues may form a rho-independent transcription terminator (Fig. 1). The ORF contains 59.0% G+C, which corresponds to the G+C content of the *P. fluorescens-Pseudomonas putida* group (34).

The deduced amino acid sequence of the ORF is very closely related to those of σ^{70} housekeeping sigma factors; it shows 83.3% identity (96.1% similarity) with RpoD of *Pseudomonas aeruginosa* (41) and 66.9% identity (91.2% similarity) with RpoD of *E. coli* (3). Further, the RpoD proteins of *Salmonella typhimurium* (8) and *Buchnera aphidicola* (25) exhibit 67.3 and 63.4% identities, respectively. Overall, housekeeping (primary) sigma factors of eubacteria have pairwise amino acid sequence identities of at least 51% (29). The ORF sequenced here satisfies this criterion and is therefore assumed to be the *rpoD* gene of *P. fluorescens* CHA0.

Sigma factors contain highly conserved regions, which are designated regions 1 to 4 (15). In region 1, which is present in the primary sigma factors (29), the RpoD proteins of *P. fluorescens* and *P. aeruginosa* have 123 identical and 3 similar amino acid residues of a total of 126 residues. Region 2, which is important for the recognition of the −10 promoter sequence and for the interaction with the core enzyme (29), shows 100% identities among the RpoD proteins of *P. fluorescens*, *P. aeruginosa*, and *E. coli* (Fig. 1). This agrees with the rule that region 2 is the most conserved polypeptide in primary sigma factors (29). Region 3, which includes a putative helix-turn-helix motif, and region 4, which contains the domain recognizing the −35 hexamer and another putative helix-turn-helix motif, are also

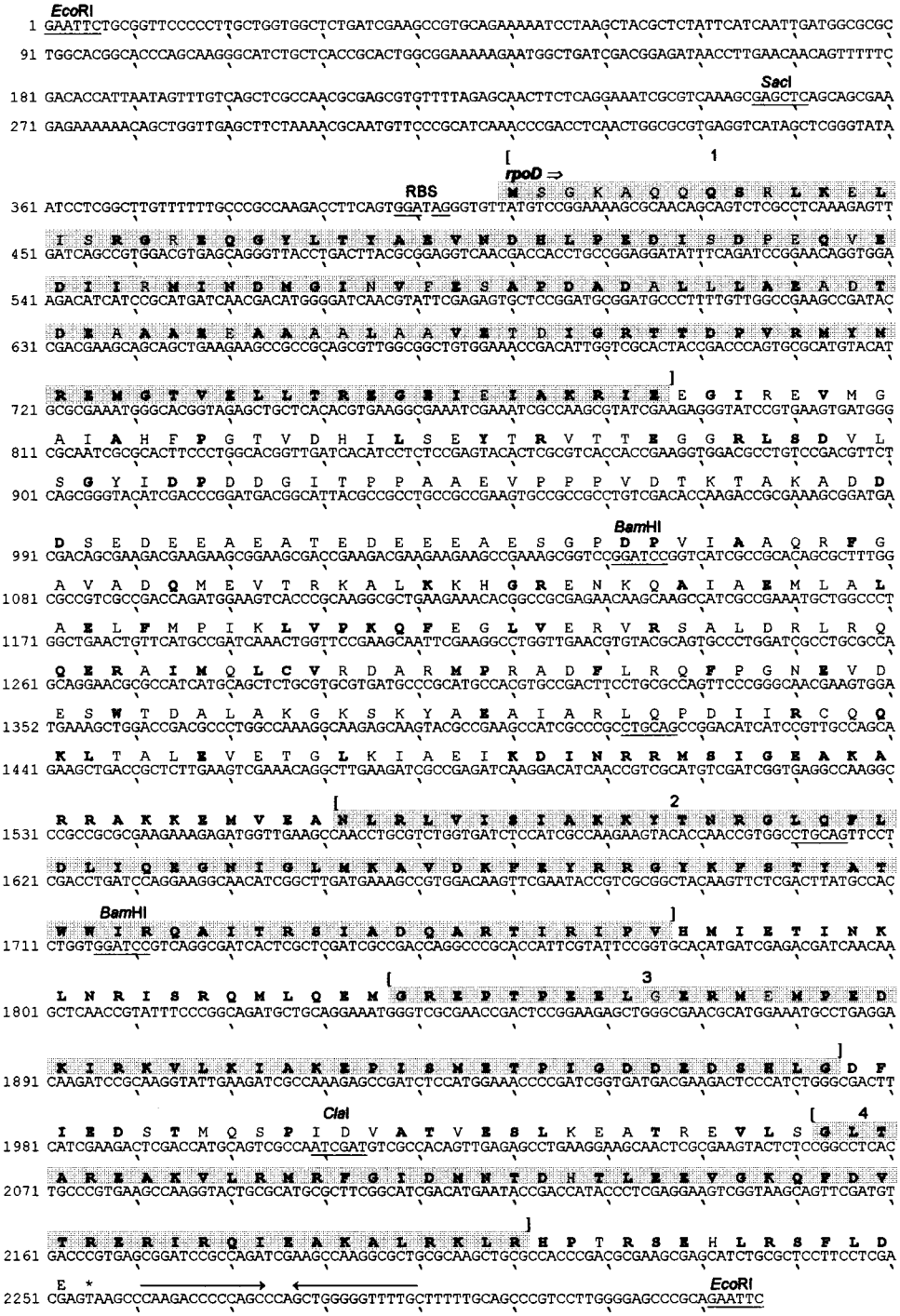


FIG. 1. Nucleotide sequence and deduced amino acid sequence of the *rpoD* gene of *P. fluorescens*. The 2.3-kb *Eco*RI fragment represents the insert in pME3415 (Table 1). The putative ribosome-binding site (RBS) is underlined. →←, inverted repeats potentially forming a transcription terminator with a predicted stability of 24.7 kcal (ca. 103 kJ) mol^{-1}. Amino acid residues which are identical in the RpoD protein of *E. coli* are in boldface type. Regions 1 to 4 (see text) are shown between brackets and indicated by a shaded background.

highly conserved among the housekeeping sigma factors (29). RpoD of *P. fluorescens* is 97.8 and 100% identical to RpoD of *P. aeruginosa* in regions 3 and 4, respectively (Fig. 1).

The deduced RpoD protein of *P. fluorescens* contains 117 acidic residues (Asp and Glu) and 98 basic residues (Lys and Arg). This large number of charged residues (35%) and retarded migration during polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) are typical characteristics of sigma factors (3, 15, 41). The *rpoD* gene of strain CHA0 was cloned into the T7 expression vector pEB16 (kindly provided by A. Darzins) and expressed in *P. aeruginosa* ADD1976 (1) by methods described elsewhere (38). The RpoD polypeptide could be visualized as an 85-kDa band by SDS-PAGE (data not shown). Thus, like other sigma fac-

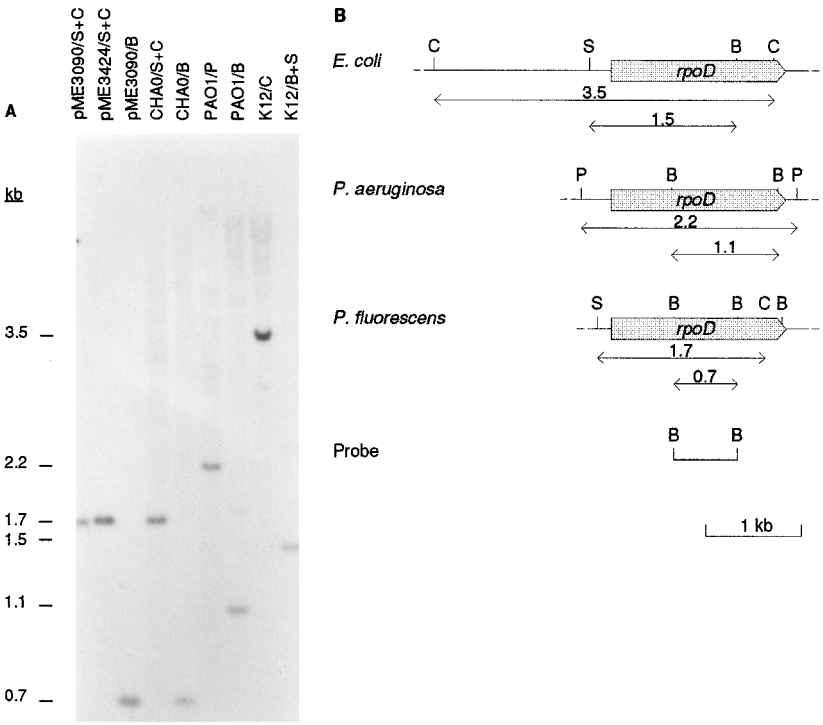


FIG. 2. (A) Southern analysis of restriction digests of total genomic DNAs of *P. fluorescens* CHA0, *P. aeruginosa* PAO1, and *E. coli* K-12 probed with a *P. fluorescens rpoD* fragment. The recombinant plasmid pME3090 and its derivative pME3424 were used as positive controls. Chromosomal DNA (≈3 μg) was extracted (12), digested, electrophoresed on a 0.7% agarose gel (48 V, 14 h), transferred to a Hybond N membrane (Amersham), and hybridized to the probe shown in panel B according to the instructions of the manufacturer (Boehringer). (B) Restriction maps of the *rpoD* locus in *E. coli* (3), *P. aeruginosa* (41), and *P. fluorescens*. Numbers above the arrows indicate restriction fragments visualized in panel A. The 0.7-kb *Bam*HI fragment marked probe was purified from low-melting-point agarose and labeled with digoxigenin-11-dUTP by using the Boehringer kit. Restriction sites: B, *Bam*HI; C, *Cla*I; P, *Pst*I; S, *Sac*I.

tors, RpoD migrated more slowly than would an average 70-kDa protein.

***P. fluorescens* CHA0 has a single, essential *rpoD* gene.** The homology of the *rpoD* gene of *P. fluorescens* with the *rpoD* genes of *E. coli* and *P. aeruginosa* was confirmed by Southern hybridization. The probe was the internal 0.7-kb *Bam*HI fragment of the *rpoD* gene of *P. fluorescens* CHA0 (Fig. 2B). This fragment contains part of the region 2 (Fig. 1). Chromosomal DNAs from *E. coli* K-12, *P. aeruginosa* PAO1, and *P. fluorescens* CHA0 were digested with restriction enzymes and hybridized with the probe under conditions of high stringency. In each case, the hybridizing bands are in agreement with the

TABLE 2. Effect of plasmids carrying *rpoD* in *P. fluorescens* CHA0 on the protection of cucumber against damping-off caused by *P. ultimum* under gnotobiotic conditions[a]

Microorganisms added[b]		Root fresh wt (mg)	Plant fresh wt (mg)	No. of surviving plants/flask	Fluorescent pseudomonads (log CFU/g)[c]
P. fluorescens	(*P. ultimum*)				
None	(−)	382 a	940 a	5.00 a	0.00 c
CHA0	(−)	386 ab	929 a	5.00 a	7.75 b
CHA0/pVK100	(−)	365 a	898 a	5.00 a	7.85 b
CHA0/pME3090	(−)	389 a	912 a	5.00 a	7.86 b
CHA0/pME3424	(−)	380 a	915 a	5.00 a	8.04 b
CHA0/pME3423	(−)	371 a	892 a	5.00 a	7.93 ab
None	(+)	44 e	150 d	0.08 d	0.00 c
CHA0	(+)	177 d	544 bc	2.93 c	8.33 a
CHA0/pVK100	(+)	146 d	400 c	2.17 c	8.05 ab
CHA0/pME3090	(+)	260 c	696 b	3.69 bc	7.91 b
CHA0/pME3424	(+)	298 bc	769 ab	3.43 bc	8.38 ab
CHA0/pME3423	(+)	335 ab	870 ab	4.28 b	8.13 ab

[a] Means within the same column followed by the same letter do not differ significantly at $P = 0.05$, according to Student's t test, when each mean is compared with each other mean and considering one independent experiment as a repetition. Each value is the mean of five individual experiments with three replicates and one flask with five plants per replicate.

[b] Bacteria and *P. ultimum* 67-1 were grown as previously described (22, 31, 48). The gnotobiotic system consisted of 1,000-ml flasks containing 400 g of sterilized artificial soil, five sterile-grown seedlings (*Cucumis sativus*), 0.2 g of millet covered with *P. ultimum*, and 10^7 CFU of bacteria per g of soil. After 2 weeks of growth (31, 32), the fresh weights of roots and shoots were determined.

[c] Log of CFU per gram of fresh root. The base-10 logarithmic transformation was applied to individual estimations of root colonization prior to statistical analysis.

restriction maps of the *rpoD* gene regions (Fig. 2A) of the strains used (3, 4, 39, 41). This experiment also provides strong evidence that a single copy of the *rpoD* gene is present in *P. fluorescens* CHA0, since the *P. fluorescens* DNA did not give any secondary (weaker) signals with the *rpoD* probe (Fig. 2A). By contrast, *Streptomyces coelicolor* and cyanobacteria have several *rpoD*-like genes (2, 5).

To check if the *rpoD* gene of strain CHA0 has an essential function, we attempted to construct an RpoD-negative mutant by gene replacement by using the ColE1-based, tetracycline-resistant suicide vector pME3087 (27, 46). We cloned the 7.0-kb *Bgl*II-*Hin*dIII fragment of plasmid pME3402 (Table 1) into pME3087 and disrupted the *rpoD* gene by insertion of the Ω-Km (kanamycin resistance) fragment (9) between the two *Bam*HI sites (Fig. 1). The resulting construct was mobilized to strain CHA0 with the derepressed conjugative plasmid R64*drd-11* and integrated into the CHA0 chromosome. Southern blot experiments showed that in two transconjugants, homologous recombination had taken place on one side, whereas in two other transconjugants the crossover was on the other side (data not shown). Excision of the vector via a second crossover was obtained by enrichment for tetracycline-sensitive cells (36). A total of 800 tetracycline-sensitive cells from three independent experiments were tested for kanamycin resistance. All tetracycline-sensitive cells were also sensitive to kanamycin, suggesting that excision invariably was a reversal of integration and that cells did not tolerate inactivation of their *rpoD* gene. The gene replacement procedure was repeated with the Ω-Km fragment inserted in the opposite orientation and with another suicide plasmid. All attempts were unsuccessful (data not shown). These results suggest that the *rpoD* gene is essential in *P. fluorescens.*

Suppression of *Pythium* damping-off of cucumber. In the presence of the pathogenic fungus *P. ultimum*, the total fresh weight and the fresh weight of roots of cucumber plants were drastically reduced, and almost no plant survived the pathogen attack (Table 2). The wild-type *P. fluorescens* CHA0 gave partial protection of the plants against the disease. Disease suppression was significantly improved when the recombinant strain CHA0/pME3090 carrying the original 22-kb insert was present (Table 2). This effect is in accordance with our previous findings (32). Protection of cucumber plants from *P. ultimum* was also improved when the plants were treated with strain CHA0/pME3424 (2.0-kb *rpoD* insert) or with CHA0/pME3423 (*rpoD* downstream of the Km promoter), and improved protection was statistically significant for root weights (Table 2). By contrast, when the vector pVK100 was introduced into CHA0 as a control, no significant difference in the disease-suppressive effect could be observed, compared with the wild-type CHA0. None of the bacterial strains had a negative effect on the growth of cucumber in the absence of the pathogen (Table 2). All *P. fluorescens* strains colonized the roots of cucumber within the same range (Table 2).

In earlier work (21, 32), growth of some plants (e.g., cress) was found to be particularly sensitive to Plt and Phl. Under gnotobiotic conditions, strains CHA0/pME3423 and CHA0/pME3424 reduced the yield of cress by about 20% in the absence of the pathogen *P. ultimum*, whereas the wild-type CHA0 had no influence on the growth of cress (data not shown). In several host-pathogen systems, strain CHA0 carrying the precursor plasmid pME3090 has given favorable biocontrol results; however, in some systems toxic side effects have been noticed on the host plants (32). Similar tests remain to be performed with the new constructs pME3423 and pME3424.

In conclusion, *P. fluorescens* CHA0 carrying pME3090, pME3423, or pME3424 synthesized more antibiotics in vitro and afforded better protection of cucumber against *P. ultimum* than did the wild-type CHA0 (Tables 1 and 2). Considering the demonstrated importance of antibiotics in biocontrol (7, 10, 13, 16, 19, 21, 23, 30, 45, 46, 50), we presume that it is the overproduction of Plt and Phl which causes the improved disease-suppressive ability of the *P. fluorescens* strains harboring an *rpoD* plasmid. In particular, a mutant of strain CHA0 which is unable to synthesize Phl and Plt does not protect cucumber from *P. ultimum* (27). However, we cannot exclude that pME3423 and pME3424 have further pleiotropic effects which might translate into plant-beneficial results.

How do multiple *rpoD* copies stimulate antibiotic production? The antibiotics Phl and Plt are typical secondary metabolites; they are synthesized by *P. fluorescens* during stationary growth phase, after several days of incubation (Table 1). Therefore, we expected that antibiotic overproduction in strain CHA0/pME3090 would be due to multiple copies of some regulatory or structural gene(s) belonging to secondary metabolism. Instead, we have found that an essential gene of primary metabolism, *rpoD*, is responsible for antibiotic overproduction by pME3090. The vector used in our experiments, pVK100 (24), has probably about seven copies in *E. coli*, and in *P. fluorescens* the copy number might be similar. Accordingly, a gene dosage effect can be expected when a gene such as *rpoD* is carried by pVK100. A gene dosage effect was manifested in strains CHA0/pME3423 and CHA0/pME3424 by increased levels of Plt and Phl (Table 1). No effect was observed on cell viability (Table 2), cell morphology, and production of another secondary metabolite, hydrogen cyanide (data not shown).

We speculate that the relative amounts of σ^{70} and σ^{38} (= RpoS, the stationary-phase sigma factor) may be particularly important in stationary phase. In *E. coli* and *P. aeruginosa*, the cellular levels of σ^{70} and σ^{38} are controlled by sophisticated regulatory mechanisms. As bacterial growth approaches stationary phase, the σ^{38}/σ^{70} ratio rises and many σ^{38}-dependent genes are expressed (11, 26, 28, 42). In another biocontrol strain (Pf-5) of *P. fluorescens*, inactivation of the *rpoS* gene stops the synthesis of the antibiotic pyrrolnitrin, a derivative of tryptophan, but enhances the synthesis of the polyketide antibiotics Plt and Phl (37). Therefore, it appears that expression of Plt and Phl depends essentially on σ^{70} and that either a lack of σ^{38} or an overdose of σ^{70} results in enhanced synthesis of Plt and Phl. In an *E. coli rpoS* mutant, some σ^{70}-dependent genes are hyperexpressed during stationary phase (33). It is conceivable that sigma factors compete for RNA polymerase and that an imbalance due to excess σ^{70} or lack of σ^{38} might enhance the expression of genes driven by weak σ^{70}-dependent promoters (33). In *P. fluorescens* CHA0, a high *rpoD* gene dose might stimulate antibiotic production directly or indirectly. In the latter case, stimulation might involve an enhanced flux of precursors (such as acetyl coenzyme A) into the polyketide antibiotic pathways. Although this interpretation is speculative at present, it would be interesting to test whether amplification of housekeeping sigma factors in industrially important microorganisms could improve the production of some antibiotics.

Nucleotide sequence accession number. The nucleotide sequence of the *rpoD* gene of *P. fluorescens* has been deposited in the EMBL, GenBank, and DDBJ nucleotide sequence data libraries under the accession number X84416.

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